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REMARKS

As indicated at the recent meeting with the Examiner, the subject matter of this application is of significant commercial importance to the assignee. In order to advance prosecution and obtain coverage for the most commercially important subject matter, Applicants have revised the present claims to be directed to specific embodiments of their invention, i.e., methods for treating secretory diarrhea by oral administration of an isolated aqueous soluble proanthocyanidin polymer composition of Croton or Calophyllum which composition is protected from the stomach environment either in enterically coated form or as a controlled release preparation. All rights are reserved to prosecute all subject matter removed from the claims in one or more subsequent applications.

The Office Action indicates that the status of the parent applications should be updated.

In compliance, the specification is amended to recite the current status of the parent applications.

Claims 1-40 and 42-75 were pending. Claims 1-20 and 48-74 have been cancelled without prejudice as non-elected. Attorneys for Applicants reserve all rights to prosecute the subject matter of non-deleted claims in a subsequent continuation or divisional application. In addition, as indicated above, 42-74 have been cancelled without prejudice as directed to a less commercially important embodiment. Claim 39 has been cancelled as redundant since claim 22 has been amended to recite administration via the oral route. Claim 21 is amended to recite a method of treating secretory diarrhea by oral administration of an isolated aqueous soluble proanthocyanidin polymer composition of Croton or Calophyllum formulated to protect the proanthocyanidin polymer composition from the stomach environment in a controlled release preparation, together with a pharmaceutically acceptable carrier. Support is found at page 11, lines 26-35; at page 20, lines 18-22; at page 21, lines 22-25. Specific support for the administration of the enterically protected isolated proanthocyanidin polymer composition as a "controlled release formulation" is provided at page 21, lines 21-24 and in the article Langer, 1990, Science 249:1527-33, (Langer) incorporated therein by reference. A copy of the Langer article is submitted herewith as Exhibit A. Attention is directed in particular to the teaching of Langer at page 1527, column 2, in particular at paragraph 2 at Figure 1D at page 1528, etc. All rights are reserved to prosecute any removed subject matter in one or more subsequent cases. New claim 76 is added to recite the method of claim 22, in which the pharmaceutical composition further comprises a pharmaceutically acceptable

carrier. Support is found in the specification, e.g. at page 20, lines 22-24; page 22, lines 37 through page 26, line 28. Upon entry of the present amendments, claims 21-38, 40, and 75-76 will be pending and under active consideration.

I. Section 112 Rejections

A. Section 112, 2nd Paragraph

Claims 21-40, 42-47 and 75 are rejected as allegedly indefinite with respect to the recitation "a pharmaceutically effective derivative thereof" because it is asserted that it is not clear what compounds are included. The claims are also alleged to be indefinite with respect to the recitation of "a therapeutically effective amount of . . . a pharmaceutically acceptable derivative thereof" as the effective amount is "not delineated with any specificity with respect to 'derivatives'."

While not agreeing in any way with these rejections and simply to advance prosecution and obtain coverage for certain embodiments, attorneys for Applicants have amended independent claims 21 and 22 (and all claims dependent thereon) to delete the recitation "or a pharmaceutically acceptable derivative thereof." Applicants specifically reserve all rights to pursue the methods of using a pharmaceutically acceptable derivative of the proanthocyanidin polymer composition in a subsequent continuation or divisional application.

Claim 32 is stated to be indefinite in reciting an improper Markush group because of the use of two conjunctive clauses.

In response, claim 32 is amended to delete the extra "and" and to be in proper Markush format. No new matter is added. In view of the above amendments, it is submitted that this rejection is avoided and must be withdrawn.

B. Section 112, 1st Paragraph

Claims 21-40, 42-47 and 75 are rejected as non-enabled for the full scope of the claims. While the Office Action admits that the specification is enabled for the treatment of travelers' diarrhea using the isolated proanthocyanidin polymer compositions, it asserts that there is no clear and specific teaching how the teaching is adapted to "treat the myriad of disease states" of diverse etiologies using each of the diverse compounds.

Attorneys for Applicants respectfully disagree. In order, however, to more clearly point out and distinctly claim the subject matter of the present claims and in no way acquiescing with this rejection and reserving all rights to prosecute any subject matter

removed from the previous claims in a subsequent continuation application, independent claims 21 and 22, (and all claims dependent thereon) are amended to recite that the methods comprise administration of an "aqueous soluble proanthocyanidin polymer composition isolated from a Croton species or a Calophyllum species." Moreover, as indicated above, merely to advance prosecution, the recitation "or a pharmaceutically acceptable derivative" is deleted from the claims.

Attention is directed to the Summary of the Invention at page 13, lines 12-37, in particular lines 12-23, which teaches that the invention encompasses methods of treating secretory diarrhea by administering a therapeutically effective amount of a proanthocyanidin polymer composition isolated from a Croton or Calophyllum species formulated to protect the proanthocyanidin polymer from the stomach environment. Attention is further directed to the Detailed Description of the Invention of the specification, in particular to Section 5.1 "Preparation of the Proanthocyanidin Polymer Composition" at page 17, line 2 through page 20, line 12 and Section 5.2. "Pharmaceutical Formulations" at page 20, line 14 through page 32, line 15. As taught at page 7, lines 9-25, the proanthocyanidin polymer composition is comprised of polymers of 2 to 30 flavanoid units; preferably is aqueous soluble and preferably is isolated from a Croton or Calophyllum species. As taught by the specification, any method known in the art can be used to isolate the proanthocyanidin polymer composition from a Croton or a Calophyllum species. See specification at page 17, line 18 through page 20, line 12. Several references describing methods of isolating the proper proanthocyanidin polymer compositions are incorporated by reference.

The specification, however, makes clear that the isolated aqueous soluble proanthocyanidin polymer composition from Croton or Calophyllum cannot be used in the methods of the present invention unless it is first formulated in a very specific way. As further taught by the specification, at page 20, lines 15-22, the present invention is based upon the Applicants' discovery that the isolated proanthocyanidin polymer composition is "labile in the environment of the stomach and is stable at pH 5.0 to approximately pH 8.0." Hence, as clearly taught, the isolated proanthocyanidin polymer composition must be formulated to protect the composition "from the acidity and enzymatic action of gastric secretions." In a preferred embodiment, the composition contains the proanthocyanidin polymer composition with an enteric coating. Specification at page 20, lines 22-25. Detailed description of enteric coatings, i.e., "those coating that remain intact in the stomach, but will dissolve and release the contents of the dosage form once it reaches the small intestine" and their use to prepare the formulations are provided at page 23, line 1 through page 30, line 23

and exemplified, e.g. in Section 8.3. As indicated above, the present claims 22-38, 40 and 75-76 are specifically directed to this embodiment.

In another embodiment, the proanthocyanidin polymer composition is protected from stomach acid and/or enzymes by formulation as a controlled release preparation. Specification at page 20, lines 18-22 and article incorporated therein by reference.

In view of the above summarized detailed teaching, it is submitted that those skilled in the art would surely be enabled to prepare the enterically protected isolated aqueous soluble proanthocyanidin polymer compositions used in the presently claimed methods.

Attention is additionally directed to the teaching of the specification in Section 2.1 of the Background of the Invention entitled "Secretory Diarrheas" and to the Detailed Description of the Invention entitled "Applications and Methods of Use" at page 32, line 17 through page 36, line 30 and to the illustrative examples in Section 7 at pages 44-50 and Section 9 at pages 59-62. In particular, as clearly taught at page 2, although associated with any of a variety of pathogenic or non-infectious etiologies, all "watery" or secretory diarrheas are "characterized by loss of fluid and electrolytes through the intestinal tract." Indeed, increased efflux of electrolytes into the intestine with osmotically drawn water resulting in loss of fluids and electrolytes characterize all forms of secretory diarrheas, regardless of etiology. Hence, agents which reduce electrolyte and fluid movement across the intestinal epithelium can be used to treat secretory diarrheas, regardless of etiology. See specification at page 3, lines 7-12. Moreover, as specifically taught in Section 5.3, at page 32, lines 18-26, the aqueous soluble isolated enterically protected proanthocyanidin polymer composition of Croton or Calophyllum species "reduces chloride flux across intestinal epithelial cells and reduces fluid movement into the intestinal lumen which results in fluid loss and dehydration associated with secretory diarrhea." Further, as demonstrated in the working example in Section 7.0 at pages 44-50, the enterically protected isolated proanthocyanidin polymer composition significantly reduced fluid accumulation in the sealed adult mouse model. Additionally, as specifically taught at page 34, lines 19-33, effective dosage ranges of the isolated enterically protected proanthocyanidin polymer compositions to be used in the presently claimed methods are in the range of 0.1 to 100 mg per day, although optimal dosages will depend upon the severity of the secretory diarrhea. In light of such detailed teaching and working examples with an art accepted animal model of secretory diarrhea, it is respectfully submitted that those skilled in the art would surely be enabled to practice the full scope of the claims as presently amended.

As indicated at the recent meeting, following the teaching of the specification, clinical trials have been conducted by administering, via the oral route, isolated aqueous soluble proanthocyanidin polymer composition of Croton in enterically coated form to patients with Travellers' diarrhea or with HIV-Associated Diarrhea. Exhibit C, submitted herewith, is a Summary of the results of these clinical trials. As demonstrated in Exhibit C at Figures 1, 2 and 3, oral administration of enterically coated isolated aqueous soluble proanthocyanidin polymer composition of Croton (designated "Crofelemer, a/k/a SP-303") to patients suffering from Traveller's or non-diarrhea produced a statistically beneficial effect including:

(1) earlier recovery time (Figure 1);

(2) increased number of patients experiencing recovery in 24 hours (Figure 2); and

(3) reduction in overall treatment failure (Figure 3).

As demonstrated in Exhibit C at Figures 4 and 6-7, in the studies of patients with HIV-Associated Diarrhea, oral administration of enterically coated tablets or beads of isolated aqueous soluble proanthocyanidin polymer composition of Croton produced clinically relevant decrease in stool weight. In addition, decreases in stool frequency and significant improvement in gastrointestinal symptoms, especially urgency were observed, particularly in patients with at least one watery stool and associated urgency at baseline.

With respect to the allegation in the Office Action that the claims are directed to treating a "multiplicity of disease states," it is respectfully submitted that this is not the present case. The claims are directed specifically to treating secretory diarrhea, i.e. excessive loss of electrolytes and fluid via the intestine associated with any of a variety of etiologies. The methods are not directed to treatment, e.g., of AIDS or cancer, but rather to treatment of the secretory diarrhea associated with such disease states.

One skilled in the art would, in view of the detailed teaching of the specification, understand such teaching and be able to use the presently taught isolated, enterically protected proanthocyanidin polymer composition to treat secretory diarrheas as presently claimed. Accordingly, the rejection of the present claims based on Section 112, first paragraph, cannot stand and must be withdrawn.

II. Section 103 Rejection

Claims 21-40, 42-47 and 75 are rejected under Section 103 as obvious in view of Ubillas et al., 1994, Phytomedicine 1: 77-106 (Ubillas) in combination with U.S. Patent No.

4,698,360 to Masquelier (Masquelier), U.S. Patent No. 5,043,160 to Würsch and Remington's Pharmaceutical Sciences (Remington's) with "applicants' admissions."

Ubillas is alleged to teach the "use of proanthocyanidin from *Croton lechleri* is old and well known" for treating diarrhea. The Office Action cites to page 78, col. 2, last paragraph. The Office Action acknowledges that this reference "differs from the claimed invention in the specific formulation and coatings." However, the Office Action alleges that "Masquelier recognizes that proanthocyanidin may be administered orally in various stable forms that include coatings" citing to col. 6, lines 43-48 and that "Würsch teaches that related tannin polymers are administered in various forms . . . including with milk . . . and as coated tablets." The specification is alleged to disclose that methods of making formulations protected against the stomach environment were known in the art as shown by Remington's. The Office Action concludes that it would have been obvious to "modify the method of treatment of Ubillas by providing proanthocyanidin formulated according to Masquelier and Würsch for "functionally and structurally related" proanthocyanidins in light of the general teachings of Remington's.

Attorneys for Applicants respectfully but emphatically disagree. Nothing in the cited combination of references suggests the presently claimed methods.

Firstly, contrary to the assertion in the Office Action, the primary reference Ubillas does not teach that isolated proanthocyanidin polymers from Croton species were used for treating diarrhea. Moreover, the proanthocyanidins described by Masquelier and Würsch are not structurally or functionally the same as those used in the presently claimed methods.

With respect to the disclosure of Ubillas, it is respectfully pointed out that the Office Action misinterprets the teaching of this reference. The Office Action asserts that the "use of proanthocyanidin from Croton is old and well known" for use in treating diarrhea citing Ubillas, page 78, col. 2, last paragraph. The cited portion of Ubillas, however, does not relate to an isolated proanthocyanidin polymer composition. Rather, Ubillas refers to the use, by native healers, of the red latex obtained from Croton trees. Ubillas teaches that the red latex commonly known as "Dragon's Blood" or "Sange de Drago" . . . .

> Its most common usages are: internally for coughs, flu 'problems with lungs', diarrhea, and for stomach ulcers; and topically as a wound healing agent for cuts, open sores, herpes infections, for the gums . . . . The general dosage for internal use is 5-10 drops of the red latex in warm or cold water, milk or in alcohol . . .

The red latex obtained from the Croton tree is surely not the same composition as the presently disclosed isolated proanthocyanidin polymer composition formulated for protection against the stomach environment. The red latex is simply the sap obtained from the Croton trees. It is a crude mixture of a larger number of different kinds of molecules produced as secondary metabolites of the trees. Attention is directed to an illustrative reference, Exhibit A, Chen et al., 1994, Planta Med 60: 541-545 "Studies on the Anti-Tumour, Anti-Bacterial, and Wound Healing Properties of Dragon's Blood" (mentioned in the specification at page 10), a copy of which is submitted herewith and which is already of record as Ref. C03). As noted on page 541, col. 2, of Exhibit A, the latex contains a "large number of secondary metabolites" including proanthocyanidins as well as six different phenols and their derivatives, three steroids and one alkaloid. There is no indication in Ubillas or in any of the cited combination of references which of the myriad of compounds in the red latex or indeed if the entire combination of compounds in the latex is active for use to treat diarrhea or any of the other myriad of conditions for which native healers use the red latex. In fact, as made clear by the Chen reference, Exhibit A, the red latex contains a variety of compounds, a number of which have biological activity.

The secondary references cited add nothing to the teaching of Ubillas to suggest use of the presently taught isolated aqueous soluble proanthocyanidin polymer compositions protected against the stomach environment.

In complete contrast to the presently used isolated, aqueous soluble proanthocyanidin polymers, which, if unprotected are degraded by the stomach environment, Würsch teaches condensed tannins which are "insoluble in cold or tepid water, and particularly at body temperature, i.e., at 37°C. The result is that they arrive in the intestine without having been degraded by gastric acid or inactivated by proteins." Würsch at col. 2, lines 24-27 (emphasis added). In fact, the disclosure of Würsch actually teaches away from and clearly indicates that, for the structurally different carob proanthocyanidins taught therein, there is no need for protection against the stomach environment.

Masquelier also does not suggest the presently claimed methods. Rather, Masquelier discloses proanthocyanidins, especially in extracts from conifers, for use against diseases associated with free radicals. With respect to formulations for oral administration, at col. 6, lines 26-28, Masquelier teaches: "For oral administration, the medicament is in the form of tablets, sugar coated pills, pellets, pills, capsules, cachets, drinkable ampoules."

There is no suggestion, much less any teaching that the extracted conifer proanthocyanidins should be protected from the stomach environment. First, there is no teaching in Masquelier that any form of the various forms enumerated for oral administration is preferred over any of the other forms. Moreover and more importantly, there is no teaching in Masquelier that any of the forms including "sugar coated pills" provide any protection of the proanthocyanidin polymers against the stomach environment. Much less is there any teaching that such protection is needed to obtain a beneficial therapeutic effect against diseases associated with free radicals, much less for therapeutic effects against secretory diarrhea.

Remington's, which, as indicated in the specification, simply provides methods for preparing formulations which are protected against the stomach environment does not supply the teaching missing from the other cited references to give those skilled in the art any expectation, much less a reasonable expectation, that an isolated, aqueous soluble proanthocyanidin polymer composition of Croton or Calophyllum would need to be enterically protected to be useful for treating secretory diarrhea.

In summary, Ubillas teaches only that the crude latex of Croton containing a myriad of different compounds has been used by native healers for diarrhea as well as for a variety of other ailments. Masquelier teaches use of conifer proanthocyanidins for treatment of diseases associated with free radicals in a variety of formulations with no suggestion to enterically protect the proanthocyanidin compositions. Würsch actually teaches aqueous insoluble proanthocyanidin compositions which do not need enteric protection as they are not degraded by gastric acid or gastric enzymes. Remington's is a standard text regarding formulations which adds nothing to the other cited references to specifically suggest that aqueous soluble proanthocyanidins should be enterically protected. Hence, it is clear that the combination of teachings from the cited references without the teaching of the present specification would not have suggested to one skilled in the art the presently claimed methods. Such hindsight attempt to use the teaching of the present specification to piece together bits and pieces of the cited references, even if unconsciously done, cannot be used to support an obviousness rejection of the present invention. See, e.g., W.L. Gore & Assoc., Inc. v. Garlock, Inc., 721 F.2d 1540, 1552 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984). Without benefit of hindsight, the combination of teachings of the cited references could not possibly and does not suggest the present methods of using aqueous soluble enterically protected proanthocyanidin polymers of Croton or Calophyllum.

In complete contrast, the present specification, e.g. at page 37, lines 5-10 teaches that the inventors unexpectedly discovered that an aqueous soluble proanthocyanidin polymer composition when orally administered could not be recovered from plasma and that the composition was quickly degraded when exposed to simulated gastric fluid but not when exposed to simulated intestinal fluids (see specification at pages 37-44).

Attention is directed to the Declaration of Dr. Akram Sabouni ("Sabouni Declaration") submitted herewith with accompanying Exhibits 1 and 2. As indicated in the Sabouni Declaration, Dr. Sabouni is one of the co-inventors of the above-identified application. Sabouni Declaration at paragraph 1. He holds a doctorate in Pharmaceutics, has post-doctoral training in Pharmaceutics and Drug Delivery and has twenty years experience in pharmaceutical research and development. *Id.* at paragraphs 2-3.

At paragraph 5 of his Declaration, Dr. Sabouni discusses the experiments presented in Section 6 of the specification at pages 37-44. As indicated by Dr. Sabouni, the experiments presented therein were conducted under his supervision to investigate whether gastric fluid or intestinal fluid had any effect on the stability of the isolated aqueous soluble proanthocyanidin polymer composition of Croton. Id.

As indicated by Dr. Sabouni, the results obtained demonstrated that isolated proanthocyanidin polymer composition was labile in the stomach environment but stable in the intestinal environment. Id. Moreover, Dr. Sabouni remarks that such results were particularly surprising and unexpected even by his colleagues who had familiarity with the isolated proanthocyanidin polymer composition. Id.

At paragraphs 5-9 of his Declaration, Dr. Sabouni discusses side-by-side experiments, performed using the murine cholera toxin (CT) treated model of secretory diarrhea described in Section 7 of the specification, at pages 44-47, using an isolated aqueous soluble proanthocyanidin polymer composition of Croton species (designated "SP-303") either enterically protected, e.g., by formulation with $NaHCO_3$ designated "SP-303+$NaHCO_3$" or without enteric protection, i.e., in water, designated "SP-303 no $NaHCO_3$."

The experiments were performed as follows:

50- to 52-day-old mice with body masses that ranged from 15.7 to 18.7 g were used. Test animals were wild type C57B1/6 and were obtained from Charles River Lab. All animals were maintained in metabolism cages with water *ad libidum* for the duration of the experiment. Mice were fasted for 24 hours prior to start of the experiment and were deprived

of food during the course of experimentation. Initially (t_0 h), mice were orally dosed with cholera toxin (CT (15μg)) and anorectally sealed with a cyano-acrylamide ester (Superglue). Three hours later (t_3 h), mice were orally dosed with an isolated, aqueous soluble, enterically protected proanthocyanidin polymer composition from Croton species (SP-303) (at a dose of 0.1, 0.3, 1.0, 3.0, 10, 25, 50 or 100 mg/kg in $NaHCO_3$) or SP-303 in water at 50 mg/kg. Control animals were dosed (at t_3 h) with either: $NaHCO_3$ alone, $NaHCO_3$ + CT or H_2O. After a 6 (t_6 h) or 7 hour (t_7 h) incubation of CT, mice were sacrificed and the entire murine small intestine from the pylorus to the rectum including cecum was isolated. Care was taken to avoid tissue rupture and loss of fluid, and the attached mesentery and connective tissues were then removed. The mass of the tissue and the fluid within was determined using an analytical balance. The tissue was then opened longitudinally, the fluid removed, and the tissue was patted dry. Fluid accumulation was measured as ratio of the mass of accumulated fluid in the intestine (small and large including cecum) versus the mass of the intestine minus the mass of the fluid. *Id.* at paragraph 6.

Exhibit 2, Table 1, presents the results from dose ranging experiments conducted with SP-303 in the cholera mouse model. When SP-303 was administered at a dose of 50 mg/kg without enteric protection, no significant reduction in fluid accumulation was observed. *Id.* at paragraph 8. In complete contrast, doses of SP-303 at 25 mg/kg and higher which were enterically protected significantly reduced fluid accumulation when compared to controls. *Id.* at paragraph 9.

Further as exemplified in the working examples in Section 7 at pages 44-50 and Section 9 at pages 59-62, the present inventors have discovered and demonstrated in working examples that the isolated, aqueous soluble proanthocyanidin polymer composition when formulated as an enterically protected, e.g., enterically coated composition significantly reduced fluid accumulation in a relevant animal model of secretory diarrhea and is useful to ameliorate stool frequency and gastrointestinal symptoms in human patients suffering from secretory diarrhea.

At paragraphs 10-11 of his Declaration, Dr. Sabouni reviews the results of experiments presented in the specification of the application in Sections 7 and 9. As concluded by Dr. Sabouni, the results presented in the specification demonstrated that the entercially protected isolated, aqueous soluble proanthocyanidin polymer of Croton species taught by the present application is useful to ameliorate frequency and gastrointestinal symptoms, *i.e.,* to treat diarrhea. *Id.* at paragraph 11.

Finally, as concluded by Dr. Sabouni, based on the experiments he has reviewed and his experience, in his opinion and judgment one skilled in the art would find the results that only when enterically protected is the isolated aqueous soluble proanthocyanidin polymer of Croton species effective to treat secretory diarrhea to be surprising and unexpected. *Id.,* at paragraph 12.

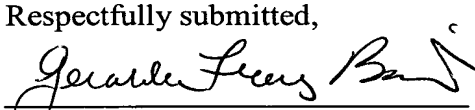
In view of these unexpected results and the remarks above, it is submitted that the present obviousness rejection cannot stand and must be withdrawn.

CONCLUSION

In light of the above amendments and remarks and the experimental evidence of unexpected results presented in the specification in Sections 7 and 9 and in Exhibit 2 of the Sabouni Declaration submitted herewith, it is submitted that all rejections have been avoided or are in error and should be withdrawn. It is further submitted that the present application is in form for allowance and early action to that end is requested.

Respectfully submitted,

Date: July 6, 2004



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28. I thank Dr. Rush D. Hole for his help in editing this article. This work was supported by United States Department of Energy Contract no. DE-AC02-76-CH03073.

New Methods of Drug Delivery

ROBERT LANGER*

Conventional forms of drug administration generally rely on pills, eye drops, ointments, and intravenous solutions. Recently, a number of novel drug delivery approaches have been developed. These approaches include drug modification by chemical means, drug entrapment in small vesicles that are injected into the bloodstream, and drug entrapment within pumps or polymeric materials that are placed in desired bodily compartments (for example, the eye or beneath the skin). These techniques have already led to delivery systems that improve human health, and continued research may revolutionize the way many drugs are delivered.

IN THE LAST FEW YEARS, WE HAVE WITNESSED AN EXPLOSION in research aimed at creating new drug delivery systems. This research has been fueled by several developments. (i) Many drugs, both old pharmaceutical products and new molecular entities, can be administered in ways that not only improve safety and efficacy but, in some cases, permit new therapies. (ii) Newer and complex drugs such as proteins are becoming available through genetic engineering; the delivery of these drugs is often more complicated than that of more conventional drugs, necessitating novel delivery systems. (iii) There is an increasing awareness that drug release patterns (continuous versus pulsatile) significantly affect therapeutic responses. (iv) The overall expense to create a pharmaceutical that is a new molecular entity is at least $150 million; the lower cost to improve the delivery of an existing drug is sometimes seen as a better investment. This issue is exacerbated because drug patents expire after 17 years, and a new drug delivery system may permit continued benefits for the company producing it. (v) Advances in materials science and biotechnology are permitting the development of new physical and chemical methods of drug delivery. In this article, some of the methods being studied to deliver drugs are discussed.

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Chemical Modification

A drug may be chemically modified to selectively alter such properties as biodistribution, pharmacokinetics, solubility, or antigenicity. One example is drugs that are designed to cross a normally impermeable barrier. The blood brain barrier, which contains tight endothelial cell junctions and prevents most molecules from entering the central nervous system, has been the target of considerable research. Several experimental approaches have been developed, in which drugs are complexed to agents that enable them to cross this barrier (for example, by rendering the drug more lipophilic or coupling it to a molecule that has a specific transport mechanism) (*1*).

Drugs have also been attached to soluble macromolecules such as proteins, polysaccharides, or synthetic polymers via degradable linkages. This process alters the drug's size and other properties, resulting in different pharmacokinetics and biodistribution. One example involves coupling the antitumor agent neocarzinostatin to styrene–maleic acid copolymers (*2*). When this complex was injected intra-arterially into patients with hepatocellular carcinoma, decreases in α-fetoprotein levels and tumor size were observed. In animals, antitumor agents such as doxorubicin coupled to *N*-(2-hydroxypropyl) methacrylamide copolymers showed radically altered pharmacokinetics, resulting in reduced toxicity. The half-life of the drug in plasma and the drug levels in the tumor were increased while the concentrations in the periphery decreased (*3*).

An exciting approach for "targeting" drugs to specific cells involves linkage of a bioactive agent (drug, radioisotope, or toxin) to a monoclonal antibody. Antibody conjugates are now being studied in the treatment of cancer, septic shock, and acquired immunodeficiency syndrome (AIDS). There are several critical issues in the use of antibodies. With mouse antibodies, anaphylactic reactions frequently occur with repeated administration. Thus, ongoing research is directed toward producing human monoclonal

antibodies or toward making mouse antibodies more human-like through the use of genetic engineering. This problem may be exacerbated for immunotoxins (antibody-toxins) because of the proteinaceous character of the toxin. Thus far, clinical usefulness of immunotoxins has been demonstrated in therapy regimens characterized by rapid pharmacokinetics, such as treatments for lymphoma and graft versus host disease, and extracorporeal treatments such as bone marrow purging. The powerful killer potential of certain toxins, such as ricin or diphtheria toxin, makes immunotoxins an attractive approach if an appropriate antibody is available that can be internalized by desired cells (*4*). Antibody-radioisotopes act over a greater distance than immunotoxins. One requirement with such complexes is the availability of a suitable chelator that allows a kinetically stable binding of the radioisotope. The degradation of the linker structure between the chelator and antibody is also critical, since nondegradable structures may cause kidney and liver toxicity. Initial clinical results with certain beta-emitters have shown regression of lymphomas. Other critical issues in the use of an antibody are its affinity, specificity, size, and large-scale production; for cancer chemotherapy, tumor characteristics and blood flow are important considerations (*5*).

Polymers, such as polyethylene glycol (PEG), can be attached to drugs to either lengthen their lifetime or alter their immunogenicity. The polymers physically prevent cells and enzymes from attacking the drug. PEG-uricase reduced serum urate levels in patients with hyperuricemia and gout; PEG-asparaginase has been used for patients with leukemia, and PEG–adenosine deaminase has been used for patients with a severe combined immunodeficiency (*6*). Drug longevity and immunogenicity may also be affected by biological approaches, including protein engineering and altering glycosylation patterns.

Vesicles

Vesicles are microparticulates or colloidal carriers composed of substances such as proteins, lipids (for example, liposomes), carbohydrates, or synthetic polymers. Vesicles share some of the advantages of drug-macromolecular conjugates (altered pharmacokinetics and biodistribution) and make possible a potentially higher drug payload. Liposomes, the most widely studied of these vesicles, can be formulated with a variety of lipid compositions and structures and are potentially nontoxic, degradable, and nonimmunogenic. However, many liposomes exhibit poor stability during storage and use. Liposome stability may be improved by increasing the liposomal cholesterol content or synthesizing polymerizable liposomes, but biodegradability may then be diminished (*7*). Engineering issues such as large-scale lipid production and manufacturing of liposomes are also critical to the more widespread use of these vesicles.

In clinical studies, liposomal doxorubicin reduces side effects such as alopecia and nausea associated with the administration of the free drug yet permits a higher maximal tolerated dose and a reduction in cardiac toxicity of 86% (*8*). Liposomal amphotericin B is more effective than the free drug in treating immunocompromised cancer patients with fungal infections (*9*). Methods are also being studied to create liposomes that release more drug in response to specific stimuli such as heat, enzymes, polycations, light, or pH (*10*).

Vesicles may be "targeted" either passively or actively. Passive targeting involves the natural uptake by cells that scavenge foreign microparticulates such as reticuloendothelial cells, which are concentrated in tissues such as the liver or spleen, or circulating monocytes. Thus, liposomes have been used for delivering toxic agents, such as arsenic, to treat liver-specific parasitic diseases (for example, schistosomiasis) in animal models (*11*) with doses 0.1% of those of conventional regimens. Similarly, immunostimulating agents encapsulated in liposomes are taken up by monocytes, which then leads to enhanced killer cell activity. This approach is being tested in certain cancer treatments (*12*). Liposomes can also be used to deliver vaccines (*13*).

Active targeting generally involves placing a charge or recognition sequence (for example, from an antibody) onto the vesicle such that it is more rapidly taken up by certain cell types (such as cancer cells) than others. One difficulty with this approach is that reticuloendothelial cells also scavenge these vesicles. However, recent approaches for altering vesicle compositions, by coating them with surfactants or altering lipid compositions, may reduce the magnitude of this problem (*14*). Vesicles that contain magnetic microparticles have also been used to target drugs to specific locations in animal models via external magnetic fields (*15*).

Controlled Release Systems

Controlled release systems deliver a drug at a predetermined rate for a definite time period. In general, release rates are determined by the design of the system and are nearly independent of environmental conditions, such as pH. These systems can also deliver drugs for long time periods (days to years). Although vesicles or drug macromolecule conjugates may prolong release, optimal control is afforded if the drug is placed in a polymeric material or pump. Controlled release systems differ from older "sustained release" or "slow release" preparations that include complexes (to salts or ion-exchange resins), suspensions, emulsions, slowly dissolving coatings that do not dissolve in the stomach yet do dissolve in the intestine (enteric coatings), and compressed tablets. Generally, sustained-release systems emit drugs in less than a day, and environmental conditions influence release rates, which leads to patient to patient variations.

Controlled release systems provide advantages over conventional drug therapies. For example, after ingestion or injection of standard dosage forms, the blood level of the drug rises, peaks, and then declines. Since each drug has a therapeutic range above which it is toxic and below which it is ineffective, oscillating drug levels may cause alternating periods of ineffectiveness and toxicity. Although sustained release preparations attenuate the peaks and valleys, they do not eliminate them. In contrast, a controlled release preparation maintains the drug in the desired therapeutic range by a single administration. Other potential advantages of controlled release systems include (i) localized delivery of the drug to a particular body compartment, thereby lowering the systemic drug level; (ii) preservation of medications that are rapidly destroyed by the body (this is particularly important for biologically sensitive molecules such as proteins); (iii) reduced need for follow-up care; (iv) increased comfort; and (v) improved compliance.

Pumps are larger and more costly than polymeric systems and require surgery for implantation; however, they offer the advantage of very precise drug control and can release the drug directly into the bloodstream. In addition, some pumps are refillable. Both externally worn and implantable pumps have been developed. In both cases, the driving force is a pressure difference, which results in bulk flow of a drug solution through an orifice.

A common externally worn pressure-driven pump is the miniature syringe pump, in which the drug is delivered at a constant rate by a syringe barrel that moves at a constant velocity; the delivery rate is adjusted by altering either the drug concentration in the syringe or the barrel velocity. An implantable pressure-driven pump has been developed that uses a fluorocarbon propellant as a driving force. In this case, the pump controls a collapsible bellows, which divides the

pump interior into two chambers, one containing the propellant and the other containing the drug solution. At body temperature, the vapor pressure exerted by the propellant forces the drug solution through a filter and flow regulator at a constant rate. Other pressure-driven pumps use piezoelectric disk benders or valves. Release rates can be externally regulated by the use of approaches such as telemetry. Pumps have been used in cancer therapy where a catheter extending from a pump is selectively inserted into a blood vessel feeding an organ such as the liver or brain to increase the delivery rate to the diseased organ while sparing the rest of the body. Pumps have also been used to release insulin, heparin, morphine, and other drugs (*16*).

Polymeric materials generally release drugs by the following mechanisms: (i) diffusion, ii) chemical reaction, or (iii) solvent activation. There are two types of diffusion-controlled systems: reservoirs (Fig. 1A) and matrices (Fig. 1B). Chemical control is accomplished either by polymer degradation (Fig. 1C) or chemical cleavage of the drug from a polymer (Fig. 1D). Solvent activation involves either swelling of the polymer (Fig. 1E) or osmotic effects (Fig. 1, F and G).

One of the first clinically used controlled release polymer systems was the Ocusert, a reservoir system designed to improve therapy for glaucoma, one of the world's leading causes of blindness. The conventional treatment involved the use of pilocarpine eye drops (which reduce intraocular pressure) four times a day. The eye drops often caused side effects, and patient compliance was sometimes poor. The Ocusert delivers pilocarpine (20 or 40 μg/hour) continuously for 1 week and controls intraocular pressure with less drug and fewer side effects. It is placed in the lower eyelid's conjunctival cul-de-sac, where it floats in the tear film. Despite its advantages, the Ocusert never achieved widespread use, initially because of its expense and poor acceptance by older patients who were reluctant to adjust to this system and later because of the introduction of timolol, a drug that requires only two applications per day.

The use of polymers to deliver contraceptive steroids has been widely studied. Four types of systems have been examined: (i) subdermal reservoir implants composed of nondegradable polymers that release drug for over 5 years (for example, the Norplant); these systems, based on a seminal study of diffusion through silicone rubber (*17*), are approved for use in 15 countries; (ii) subdermal implants or injectable microspheres composed of degradable materials, such as lactic acid–glycolic acid copolymers, polycaprolactones, or cholesterol; (iii) steroid-releasing intrauterine devices, such as the Progestasert, an ethylene–vinyl acetate copolymer reservoir that contains a 3-day supply (38 mg) of the amount of progesterone normally taken orally, but which, since it delivers progesterone to its

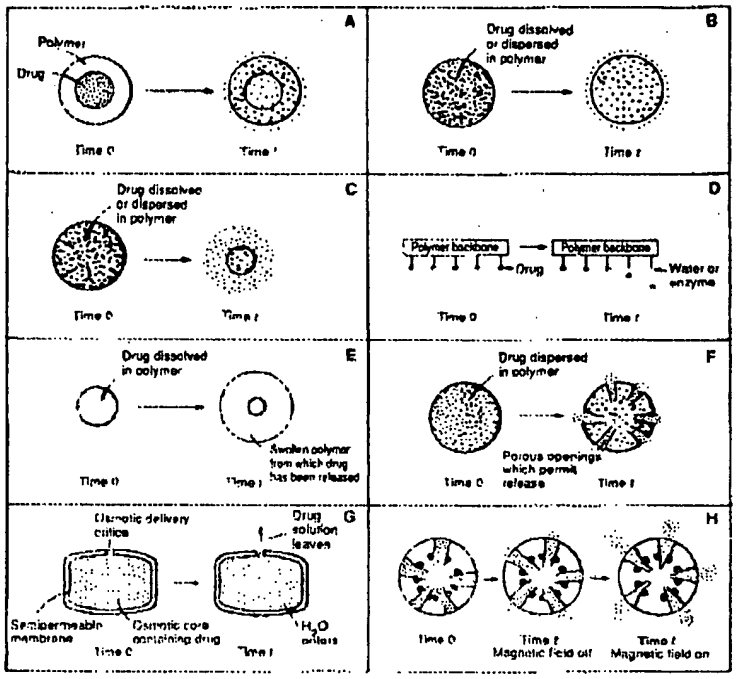

Fig. 1. Polymer release mechanisms. The most common release mechanism is diffusion, whereby the drug migrates from its initial position in the polymeric system to the polymer's outer surface and then to the body. Diffusion may occur through a reservoir (**A**), in which a drug core is surrounded by a polymer film, or in a matrix (**B**), where the drug is uniformly distributed through the polymeric system. Drugs can also be released by chemical mechanisms such as degradation of the polymer (**C**) or cleavage of the drug from a polymer backbone (**D**). Exposure to a solvent can also activate drug release. For example, the drug may be locked into place by polymer chains, and, upon exposure to environmental fluid, the outer polymer regions begin to swell, allowing the drug to move outward (**E**), or water may permeate a drug-polymer system as a result of osmotic pressure, causing pores to form and bringing about drug release (**F**). An attractive osmotic system that can provide constant release rates exists in the form of a pill that has a laser drilled hole in the surface of a polymer coating (**G**). Some polymer systems can be externally activated to release more drug when needed, using forces such as magnetism (**H**). In this case, an external magnetic field causes polymer-embedded magnetic beads to "bounce" drug-containing pores, forcing more drug out of a matrix. In all cases, dots represent drug, and in (**H**) the larger dots represent magnetic beads. Combinations of the above mechanisms are possible. Release rates from polymer systems can be controlled by the choice of the polymeric material (for example, crystallinity or core structure for diffusion-controlled systems; the lability of the bonds in the backbone of the monomers for chemically controlled systems; and the design of the system (for example, thickness and shape). The advantage of having systems with different release mechanisms is that each can accomplish different goals. For example, reservoir systems are able to produce near constant release rates, whereas matrix systems are inexpensive to manufacture. Chemically controlled systems generally result in elimination of the polymer, whereas solvent-activated systems have release rates independent of pH (*50*).

target locally at a rate of approximately 65 μg/day, lasts for over 1 year; and (iv) vaginal rings, which are silicone reservoir systems used for 3 to 6 months; generally, for each monthly cycle they are inserted for 3 weeks and then are withdrawn for 1 week (*18*).

Tetracycline, incorporated into diffusion-controlled systems composed of ethylene–vinyl acetate copolymer or other substances, has been used to treat periodontal disease. When this controlled release system was placed in the periodontal pocket, significant reductions in bacterial counts and in the incidence of gingivitis were observed. Furthermore, because the systems are placed next to their target, treatment is accomplished with less than one-thousandth of the normal systemic dose (*19*).

A number of other controlled release systems are under study. These include localized release of diphosphonates (calcium chelators) to prevent heart valve calcification, dopamine or bromocriptine for potential treatment of Parkinson's disease, and bethanecol for potential treatment of Alzheimer's disease (*20*).

Controlled Release Systems for Peptides and Proteins

For many years, controlled-release systems were capable of slowly releasing drugs of only low molecular weight (<600). Large molecules such as proteins were not considered feasible candidates, because polypeptides were considered too large to slowly diffuse through most polymeric materials, even after swelling of the polymer. Large molecules could diffuse through highly porous membranes such as Millipore filters or certain gels such as polyacrylamide; however, in these cases, diffusion was generally too rapid to be of value and tissue damage was usually observed. The discovery that matrices of solid hydrophobic polymers containing powdered macromolecules enabled molecules of nearly any size to be released for over 100 days permitted controlled delivery of a variety of proteins, polysaccharides, and polynucleotides (*21*). Examples of polymers that perform in this way are nondegradable ethylene–vinyl acetate copolymer and degradable lactic acid–glycolic acid copolymers. Certain hydrogels such as poly(hydroxyethylmethacrylate) or poly(vinylalcohol) also work effectively but release proteins for shorter time periods than the above polymer systems.

The release mechanism generally involves movement of the polypeptide through a complex porous path in the polymer matrix. If the polymer erodes, this will affect the pore structure and accelerate the release. Factors influencing release rates include protein particle size and loading, protein solubility and molecular weight, polymer composition and molecular weight, and the dimensions and shape of the matrix (*22*). Polymer systems are now being used in animal studies to release many proteins, including insulin, growth factors, and angiogenesis inhibitors (*23*). The first Food and Drug Administration (FDA)–approved system for controlled release of a peptide, the Lupron Depot (injectable microspheres composed of lactic acid–glycolic acid copolymer and leuprolide acetate, and lasting 30 days) was recently introduced for the treatment of prostate cancer. Other polymeric systems for releasing similar drugs (*24*) are also under evaluation for treating endometriosis and other conditions.

A number of challenges in protein delivery remain. Foremost among these is that, when encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C. This can cause a loss of biological activity and possible changes in immunogenicity. Stabilization approaches being developed in protein chemistry (*25*) will be important for the success of some of these delivery systems. In one study that used solid proteins as a model, small amounts of added water induced aggregation of albumin, ovalbumin, glucose oxidase, and β-lactoglobulin. The aggregation as a function of added water went through a maximum with just 3 μl of water, causing 97% aggregation of 10 mg of albumin in 24 hours. At lower and higher water concentrations, aggregation was reduced. The aggregation mechanism was discovered to be intermolecular S–S bond formation through thiol-disulfide interchange. This, in turn, suggested rational strategies for protein stabilization, including modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions (*26*). In a study of ribonuclease, oxygen was responsible for protein aggregation (*27*).

Transdermal Controlled Release Systems

The skin is often considered a barrier that keeps all agents, including drugs, out of the body. However, a few drugs have just the right properties to penetrate the skin at appreciable rates and are potent enough so that only low doses are required. Furthermore, compared to the oral route, losses due to liver metabolism are reduced. The rate-limiting barrier to drug entry through the skin is the outermost skin layer, the stratum corneum, which is composed primarily of keratin and lipids. For a drug to penetrate the skin significantly, it should have a low molecular weight and appreciable solubility in both water and oil.

The first transdermal delivery system introduced clinically released scopolamine from patches (reservoir systems) to prevent nausea associated with motion sickness. After the patch has been applied, a 4- to 6-hour lag period is required for the drug to reach therapeutic concentrations. Because of the small amount of drug required (*7* μg/hour over 3 days) and the high skin permeability of scopolamine, this system can be designed so that the device rather than the skin is rate-controlling. This minimizes patient to patient variations. The device is placed behind the ear because the permeability of the stratum corneum there is comparatively high, which further enables the device, rather than the skin, to provide the principal diffusion barrier.

The most widely used transdermal systems release nitroglycerin daily for the treatment of heart disease. These systems, first introduced in 1982, have annual sales of approximately $500 million. The amount of nitroglycerin absorbed is determined by the skin rather than the device; nitroglycerin patches of different sizes are available so that patients can select the desired dosages. However, the continuous delivery of nitroglycerin may create drug tolerance. The possibility of controlled intermittent delivery of nitroglycerin is being explored.

A weekly clonidine patch and a twice weekly estradiol patch are used to treat hypertension and estradiol deficiency (for postmenopausal females), respectively. There have been reports of local irritation with these systems, perhaps because of their longer application periods or because of the combined effects of bioadhesives, chemicals, and drugs used in the formulations. Transdermal systems for the delivery of testosterone, fentanyl, isosorbide dinitrate, nicotine, timolol, and antihistamines, although not yet clinically available, are under study.

The biggest challenge in transdermal delivery is to increase the variety of drugs that can be administered. Four approaches have been explored. Electrical means such as iontophoresis, which can drive charged molecules through the skin, have received considerable attention. It has been proposed that iontophoresis might allow the transdermal delivery of larger molecular weight drugs, such as insulin. Animal studies with insulin have not led to conclusive results; insulin permeation depends on the animal model, the type of

current, and whether the stratum corneum has been removed (*28*). Nonetheless, clinical studies have shown that smaller peptides such as luteinizing hormone–releasing hormone (LHRH) can be delivered at increased rates (*29*). A second approach uses ultrasound to enhance transdermal drug permeation. Ultrasound also eliminates the lag times associated with transdermal drug delivery in animal models (*30*). Chemical modification provides a third approach: a lipophilic drug could be synthesized that penetrates the skin and is subsequently converted by epidermal enzymes into the original drug. Finally, penetration enhancers such as Azone, dimethyl sulfoxide, and dimethyl formamide have been used. However, extensive testing must be done to establish safety. It may be more useful to utilize agents used in FDA-approved topical formulations (for example, ethanol is used in the estradiol system to enhance penetration).

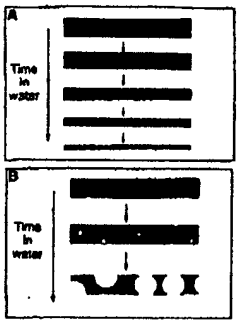

Fig. 2. Idealized diagram of polymer matrices displaying surface erosion (**A**) or bulk erosion (**B**).

Novel Degradable Polymers

Most materials used in medicine today were not designed for biomedical applications. For example, the polymers used in the artificial heart and dialysis tubing were originally used in ladies' girdles and sausage casings, respectively. These materials were chosen because they appeared, to some extent, to resemble the organs they were intended to replace. A significant challenge is to develop more rational approaches for creating improved materials for humans. This may be particularly important in the development of degradable polymers.

For such polymers, to maximize control over release, it is often desirable for a system to degrade only from its surface (Fig. 2A). [The only degradable polymers in common use, polyesters such as lactic acid–glycolic acid copolymers, display bulk (homogeneous) erosion (Fig. 2B), resulting in significant degradation in the matrix interior.] For surface-eroding systems, the drug release rate is proportional to the polymer erosion rate. This eliminates the possibility of dose dumping, improving device safety; release rates can be controlled by changes in system thickness and total drug content, facilitating device design. Achieving surface erosion requires that the degradation rate on the polymer matrix surface be much faster than the rate of water penetration into the matrix bulk. Efforts have begun to design such ideal polymers. Theoretically, the polymer should be hydrophobic but should have water-labile linkages connecting monomers.

It was proposed that, because of the lability of anhydride linkages, polyanhydrides would be a promising class of polymers. By varying the monomer ratios in polyanhydride copolymers, surface-eroding polymers lasting from 1 week to several years were designed and synthesized (*31*).

The possibility of implanting polyanhydride disks containing nitrosoureas for treating brain cancer after surgery is being explored. Normally, nitrosoureas are given intravenously (they have a half-life of 12 to 15 min and cause serious toxicity to several organs). By placing nitrosoureas in polyanhydrides, the drug is protected and its efficacy lasts approximately for the duration of the polymer lifetime (in this case, nearly 1 month). The polymer disks also deliver the drug locally to the brain, significantly reducing systemic toxicity. Surface erosion is desirable, for, if bulk erosion were to occur, uncontrolled amounts of this potentially toxic drug could be released during breakup of the matrix. These polymers have been shown to be safe in numerous animal models (*32*). Institutional Review Board approval was then obtained to conduct clinical trials with polyanhydrides at five U.S. hospitals. In 1987, the FDA approved these polyanhydrides for clinical trials. In an initial study of 21 patients, safety was demonstrated and patient lifetime was extended significantly beyond that afforded by conventional treatments (*33*). A phase-3 trial involving 32 hospitals is currently under way; over 100 patients have been treated.

Several different surface-eroding polyorthoester systems have been synthesized. In this case, additives are placed inside the polymer matrix, which causes the surface to degrade at a different rate than the rest of the matrix. Such a degradation pattern can occur because these polymers erode at very different rates, depending on pH, and the additives maintain the matrix bulk at a pH different from that of the surface. By varying the type and amount of additive, release rates can be controlled (*34*).

It may be desirable to have degradable polymers that consist of, and break down into, naturally occurring metabolites. Thus, new polyamino acids were synthesized in which L-amino acids or dipeptides were polymerized by nonamide bonds between functional groups (for example, esters) located on amino acid side chains. This approach permits the synthesis of biomaterials (for drug delivery systems, artificial organs, vascular grafts, or other prostheses) that are derived from nontoxic substances, which also have other desirable properties: (i) the incorporation of an anhydride linkage into the polymer backbone causes rapid degradability; (ii) an ester bond provides better film and fiber formation; and (iii) an imide or iminocarbonate bond improves mechanical strength (*35*).

One such polymer is being studied in vaccine delivery. Many adjuvants such as aluminum oxide or Freund's adjuvant rely on a simple "depot" effect, releasing antigen over a short period, from several hours to a few weeks. In earlier studies in mice and rabbits, prolonged release of small amounts of antigen from a nondegradable device resulted in sustained antibody production for over 6 months (*36*). Although these studies demonstrated the potential value of controlled release in immunization, it would be advantageous to use degradable systems to avoid implant retrieval. This concept is particularly attractive, because the polymer degradation products could be intentionally designed to have adjuvant properties, that is, an "engineered" polymer. This would permit the design of a system that could stimulate the immune response while simultaneously releasing antigen over long periods. Because of the adjuvanticity of L-tyrosine and its derivatives, a polymer consisting of tyrosine or a tyrosine derivative connected by hydrolyzable iminocarbonate bonds was synthesized. When this polymer was converted into small pellets, this system provided sustained adjuvanticity while simultaneously serving as an antigen repository. The release of antigen from a single tyrosine-based polyiminocarbonate pellet gave rise, in mice, to higher antibody titers than release of the same antigen dose from a control polyiminocarbonate pellet or from two injections of the antigen over 1 year (*37*).

Pulsatile Polymeric Controlled Release Systems

It would be desirable if polymeric systems could be designed to release increased levels of drug when needed; this would mimic the body's physiological processes. Both open-loop and closed-loop approaches are being studied. One open-loop system contains drug and small magnetic beads embedded in a polymer matrix (Fig. 1H). Release rates are enhanced when desired by an oscillating external magnetic field. Parameters that affect the release rate include the magnetic field frequency and strength, the polymer composition, and the strength and orientation of the polymer-embedded magnets. Application of the magnetic field causes up to 30-fold increases in release rates (*38*). Ultrasound can also be used to enhance drug release rates from polymers (*39*). Successful clinical implementation of the ultrasonic or magnetic systems will probably require the creation of small portable triggering devices (wristwatch size) that can be preprogrammed or activated manually when desired.

Several closed-loop polymeric systems are being developed. In one case intended for the increased release of insulin in the presence of excess glucose, glucose oxidase was immobilized within an insulin-containing polyamine membrane. Glucose oxidase converts glucose to gluconic acid; the acid protonates amine groups within the membrane. The electrostatic repulsion of the positively charged amine groups causes expansion of the membrane and increased delivery of insulin. As the physiologic glucose concentration decreases in response to the released insulin, the membrane contracts, decreasing the rate of insulin release (*40*). In another approach, glucose oxidase was immobilized to agarose beads contained within a polymer matrix. The acid formed when external glucose reacts with the immobilized enzyme lowers the pH, which changes the solubility of insulin and the diffusional driving force. Increased release rates to glucose challenges were observed in vitro and in diabetic rats (*41*). A third approach involves the synthesis of glycosylated insulin bound to concanavalin A (Con A). Con A is immobilized on Sepharose beads. The glycosylated insulin is displaced from Con A in response to glucose, which competes for the same binding sites. The rate of insulin release also depends on the binding affinity of the insulin derivative to Con A and can be influenced by the choice of saccharide group in glycosylated insulin. By encapsulating glycosylated insulin-bound Con A within a suitable polymer that is permeable to both glucose and insulin, it is possible to control glucose influx and insulin efflux (*42*). Critical issues with respect to each of these delivery systems are the stability of insulin and enzymes and the rapidity of movement (response time) of insulin from the polymer matrix to the circulation. Such systems may also benefit from ongoing research in biosensors (*43*).

Research is being conducted on self-triggered release of drugs such as narcotic antagonists in multicomponent systems involving erodible polymers, antibodies, and enzymes (*44*). Pulsatile systems involving pH-sensitive or temperature-sensitive polymers are also being studied, as are polymer systems that can be activated by light or electricity (*45*).

Conclusions and Future Directions

The studies discussed here show that carriers can affect drug level, location, longevity, and antigenicity. Although this technology is at an early stage, it has already made a significant clinical and commercial impact. This technology is not limited to medicine. Controlled release has been used for pet flea collars, pesticides, anti-fouling agents, fertilizers, and fragrances. Liposomes are used in cosmetics.

There are numerous challenges ahead. One area is the creation of bioadhesive polymers that could alter a drug's location when given orally (*46*). This could be particularly important for drugs that are absorbed only in certain segments of the gastrointestinal tract. Even more tantalizing, but more difficult, is delivering large and complex molecules such as proteins orally. Research on novel anatomical delivery pathways such as the nose or lung may also permit the delivery of a wider spectrum of drugs. Furthermore, an understanding of cell transport mechanisms may aid in cellular targeting (*47*).

Although this article has focused principally on specific carriers for pharmaceuticals, ongoing research in cell transplantation could be used to provide desired agents (*48*). The possibility of inserting genes into cells to produce desired entities is being explored (*49*).

Furthermore, continuous advances in biotechnology will have at least several major effects on drug delivery. First, novel complex drugs will be created that will be difficult to administer by conventional means. Second, approaches being developed in genetic engineering may enable the creation of new molecular constructs (for example, deletion mutants, hybrid proteins, and ligated gene fusion hybrids) with increased ability to achieve site-specific delivery. Finally, advances in materials science and chemical engineering should permit improved polymers, lipids, antibodies, and other substances to be synthesized, better understood, manufactured, and effectively used in drug delivery.

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51. I thank H. Bernstein, S. Cohen, E. Edelman, A. Klibanov, and B. Tomlinson for their helpful reviews of this manuscript and J. Folkman for providing me with the inspiration to do research in this field. Supported by NIH grant GM 26698.

Molecular Targets for AIDS Therapy

HIROAKI MITSUYA, ROBERT YARCHOAN, SAMUEL BRODER

The development of antiretroviral therapy against acquired immunodeficiency syndrome (AIDS) has been an intense research effort since the discovery of the causative agent, human immunodeficiency virus (HIV). A large array of drugs and biologic substances can inhibit HIV replication in vitro. Nucleoside analogs—particularly those belonging to the dideoxynucleoside family—can inhibit reverse transcriptase after anabolic phosphorylation. 3′-Azido-2′,3′-dideoxythymidine (AZT) was the first such drug tested in individuals with AIDS, and considerable knowledge of structure-activity relations has emerged for this class of drugs. However, virtually every step in the replication of HIV could serve as a target for a new therapeutic intervention. In the future, non-nucleoside-type drugs will likely become more important in the experimental therapy of AIDS, and antiretroviral therapy will exert major effects against the morbidity and mortality caused by HIV.

HUMAN IMMUNODEFICIENCY VIRUS (HIV) IS A PATHOGENIC RETROVIRUS and the causative agent of acquired immunodeficiency syndrome (AIDS) and its related disorders. One of the central questions after HIV was discovered was whether antiretroviral therapy would even be feasible. Since that time, one drug, 3′-azido-2′,3′-dideoxythymidine (AZT or zidovudine) (1) has been shown to prolong the survival and improve the quality of life of individuals with advanced HIV infection (2–4). More recently, the administration of AZT was shown to delay clinical progression in certain asymptomatic individuals with HIV infection (4). Thus, the central question now is no longer whether antiretroviral therapy will be feasible, but rather, how to use the emerging knowledge of the viral life cycle to create new opportunities for therapy.

The purpose of this review is to discuss some principles for the development of antiretroviral drugs in the therapy of HIV infection and to highlight some recent advances in this area. Successful antiviral drugs, in theory, exert their effects by interacting with viral receptors, virally encoded enzymes, viral structural components, viral genes or their transcripts, or cellular factors required for viral

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Studies on the Anti-Tumour, Anti-Bacterial, and Wound-Healing Properties of Dragon's Blood[1]

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[1] Part 4 in the series "Biological and Chemical Investigation of Dragon's Blood from Croton Species of South America". For Part 3, see Ref. (10)

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Received: January 17, 1994; Revision accepted: March 13, 1994

Abstract

Three *in-vitro* assays have been adopted to examine the cytotoxicity and anti-bacterial activity of the blood-red sap of *Croton lechleri* from Ecuador, and to examine its effect upon the proliferation of endothelial cells. The sap was found not to be cytotoxic. Several simple phenolic compounds and diterpenes showed a potent anti-bacterial activity. The sap has little effect upon the proliferation of endothelial cells, and no single active ingredient was identified. A mechanism for the wound-healing property of the sap has been proposed.

Key words

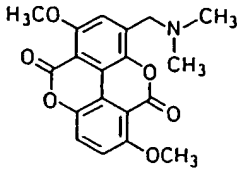
Croton spp., Euphorbiaceae, Dragon's blood, Sangre de Grado, cytotoxicity, anti-bacterial, endothelial cells.

Introduction

Dragon's blood, also known as Sangre de Draco or Sangre de Grado, is the dark-red viscous sap produced by several *Croton* species, such as *C. lechleri* and *C. draconoides*. These plants are mainly distributed in South American countries, including Peru and Ecuador. The sap has long been used in those countries as a folk remedy, and claimed to be effective for the treatment of a variety of illnesses and diseases, amongst which are wounds, cancer, inflammation, and infection (1–3). It is available commercially and may be taken orally or applied externally. Because of its popular use, the existence of these trees is threatened since the trees are normally felled in order to collect the sap.

Despite its popular applications, however, until very recently, there was little knowledge of the chemical composition of the sap, and its biological properties were scarcely known. The anti-inflammatory activity (4) and wound healing properties (5) of the sap have been attributed to the phenanthrene alkaloid, taspine (1). This latter finding has been disputed and the lignan, 3′,4-*O*-dimethylcedrusin (2) has been proposed as the active principle (6). It has been demonstrated that taspine has potent activity against KB cells and V-79 cells and may be responsible for the anti-cancer activity of the sap (7).

The sap of *Croton lechleri* has been subjected to a detailed chemical and biological study in our laboratory. It has been shown that the sap was an aqueous solution containing a large number of secondary metabolites, and that proanthocyanidin(s) of varying molecular sizes were its major constituents (> 90% of dry weight) (8). Amongst those minor constituents, six simple phenols and their derivatives, three steroids and one alkaloid were identified (9). The examination of the constituents of the bark led to the characterisation of six diterpenoids, four of which were novel diterpenoids, and these diterpenoids were also found in the sap in a very small quantity (9, 10).



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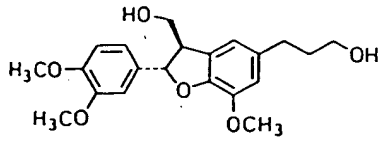

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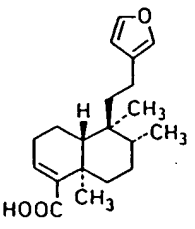

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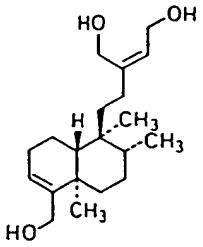
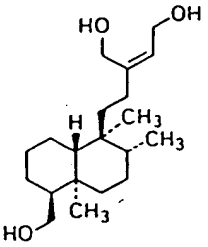


4 bincatriol



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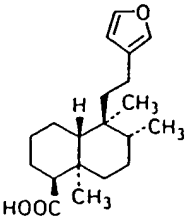

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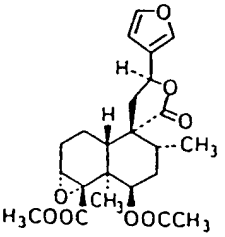
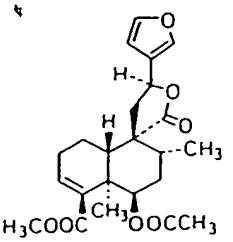


7 korberin A



8 korberin B

Planta Med. 60 (1994) 541–545

The biological activities of the sap have been evaluated by a number of *in-vitro* and *in-vivo* assays. In relation to its anti-tumour, anti-infections, and wound-healing properties, we have examined cytotoxicity and anti-bacterial activity of the sap and its effect on the DNA synthesis of endothelial cells, and the details are reported in this communication.

Materials and Methods

Samples and extracts

The sap of *C. lechleri* L. and other parts of the plant were kindly provided by Professor M. H. Zenk (Universität München) and Professor Y. Gleba (Academy of Science, Ukraine), who collected them in Ecuador in December, 1989.

A small portion of the sap (10 ml) was freeze-dried as the dried sap sample. The sap (500 ml) was extracted successively with chloroform (5 × 500 ml) and ethyl acetate (3 × 500 ml), and the chloroform-soluble and ethyl acetate-soluble fractions were obtained (0.45 g and 18.6 g, respectively). A large volume of acetone (1000 ml) was then added to the residual aqueous solution to give the acetone-soluble fraction (44.1 g). The insoluble materials were dissolved in methanol (500 ml), and removal of the residue gave the methanol-soluble fraction (12.8 g).

The bark (530 g) was ground to fine powder and extracted with methanol (3 × 1000 ml). After being concentrated to a small volume, the extracts were suspended in distilled water (100 ml), and extracted with chloroform (3 × 200 ml) to give a chloroform-soluble fraction (23 g).

Isolation and characterisation

All the fractions were separated by chromatographic methods (silica gel for the two chloroform fractions and Sephadex LH-20 for the other fractions). The details of the separation, purification, and characterisation of the constituents were described in our earlier communications (8–10). The pure compounds involved in this work were (+)-catechin, (+)-gallocatechin, (−)-epicatechin, (−)-epigallocatechin, procyanidin B-4 (dimer), proanthocyanidin trimer, proanthocyanidin tetramer, proanthocyanidin heptamer, 1,3,5-trimethoxybenzene, 2,4,6-trimethoxyphenol, 4-hydroxyphenethyl alcohol, β-sitosterol, β-sitosterol-β-D-glucopyranoside, hardwickiic acid (3), bincatriol (4), crolechinol (5), crolechinic acid 6), korberin A (7), and korberin B (8) (8–10).

Evaluation of cytotoxicity

The cytotoxicity of the samples was evaluated using the microdilution method developed by Anderson et al. (11), but with slight modification. KB cells derived from human oral epidermoid carcinoma were purchased from ICN Flow Laboratories (UK) and routinely maintained at 37 °C with 5 % CO_2 in Eagle's minimum essential medium with Eagle's salts and sodium bicarbonate (0.85 g/l) (ICN Flow Laboratories, UK), and supplemented with non-essential amino acids (1 %), foetal bovine serum (10 %), L-glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml). Prior to use, cells were harvested with trypsin/EDTA (6–8 ml/flask), and diluted with the growth medium to the required concentration.

KB cells (2.5×10^4 per well in a 96-well microtitration plate) were exposed to 7 two-fold serially diluted concentrations of each sample (an extract or a pure compound) at 37 °C with 5 % CO_2. The total volume of solution in each well was 100 μl. After incubation for 48 hours, the cells in each well were fixed with an aqueous trichloroacetic acid solution (10 % v/v) at 4 °C for 1 hour, and then rinsed with distilled water 5 times and left to dry. The cells were then stained with 1 % aqueous eosin B stain (Sigma) for 1 hour, and washed 3 times with diluted acetic acid (1 %, v/v). The dye in each well was extracted with an aqueous NaOH solution (5 mM, 200 μl) for 30 minutes and the optical density (O.D.) at 490 nm of the solution in each well was measured on a MR700 microplate reader (Dynatech Labs. Inc., UK). Triplicate determinations of cytotoxicity for each sample were carried out on three separate occasions. Two controls were simultaneously set up to monitor the accuracy of the assay throughout the experiment: one blank as a negative and emetine chloride as a positive control. The IC_{50} value for emetine chloride was 0.2 ± 0.03 μg/ml (mean ± SD, n = 26). All the samples examined were dissolved in ethanol and diluted with the cell culture medium with the final concentration of ethanol being less than 0.1 %.

Bioautographic TLC assay of antibacterial activity

A direct bioautographic TLC assay developed by Hamburger and Cordell (12) was used in this study with slight modifications. *Bacillus subtilis* (strain JTS 13, from Professor J. T. Smith's collection held at The School of Pharmacy) and *Escherichia coli* (strain KL 16) were grown on nutrient agar plates at 37 °C. The agar plates were prepared from the medium containing Lab M agar (1.5 % w/v) and nutrient broth No 2 (Oxoid, 2.5 % w/v). A single isolated colony from a fresh nutrient agar plate was aseptically transferred to the nutrient broth and incubated at 37 °C for 18 hours. A suspension of 6×10^8 bacteria/ml was then prepared for the assay. The samples were dissolved in appropriate solvents and spotted with varying concentrations on TLC plates (silica gel G60 F254 A1, 20 × 20 cm). As the TLC plates were completely dried, the bacterial suspension was evenly spread using a soft brush over the plates, which were incubated in humidified plastic boxes at 37 °C for 24 hours. The plates were then sprayed with an aqueous solution of *p*-iodonitrotetrazolium (4 mg/ml, 6 ml per plate) and incubated again at 37 °C for 4 hours. The plates were then treated with a 70 % aqueous ethanol solution. Metabolically active bacteria convert *p*-iodonitrotetrazolium to intensely coloured formazan. Thus zones of inhibition of bacterial growth appear as clear spots against a coloured background. Both penicillin V and chloramphenicol were used as positive controls.

Effect on DNA synthesis of endothelial cells

Bovine endothelial cells (BAE-1) were obtained from European Collection of Animal Cell Culture (UK) and maintained at 37 °C with 5 % CO_2 in Dulbecco's modified Eagle's medium/nutrient mix F12 (Gibco, UK) containing fetal calf serum (10 %) and penicillin/streptomycin. The endothelial cells were seeded in 24-well plates at the density of 6×10^4 cells per well and incubated for 48 hours. The cell culture medium was then changed and the content of serum in the medium was reduced to 2.5 %. After incubation for another 24 hours, ^3H-thymidine (1 μCi/ml), together with a solution of the sample, was added to the cell, and the plates were further incubated for 8 hours. The cells were rinsed with Hank's balanced salt solution (HBSS) and fixed in ice-cold 10 % trichloroacetic acid for 20 min. The DNA of the cells was extracted using 2 N perchloric acid at 60 °C for 30 min, and the solution was collected and counted on a Beckman LS6000TA scintillator.

Results and Discussion

In-vitro *cytotoxicity*

The sap, its various extracts, and individual compounds were evaluated by the KB cell assay, and their cytotoxity is presented in Table 1.

The IC_{50} value for the original sap was greater than 900 μg/ml, and when it was dried, the IC_{50}

Table 1 Cytotoxicity of the sap evaluated by *in vitro* KB cell assay.

Sample	IC_{50} (μg/ml)
Emetine chloride (control)	0.2 ± 0.03 (n = 26)
Sap	> 900
Dried sap	187
$CHCl_3$ extract	186
EtOAc extract	70
Acetone extract	125
MeOH extract	185
MeOH extract (bark)	50
MeOH extract (heartwood)	25
MeOH extract (leaf)	90
(+)-Catechin	> 235
(+)-Gallocatechin	> 450
(−)-Epicatechin	> 250
(−)-Epigallocatechin	> 35
Procyanidin B-4 (dimer)	> 250
Proanthocyanidin trimer	> 175
Proanthocyanidin tetramer	> 50
Proanthocyanidin heptamer	> 50
1,3,5-Trimethoxybenzene	7.13 ± 1.70
2,4,6-Trimethoxyphenol	> 25
4-Hydroxyphenetheyl alcohol	> 50
β-Sitosterol	20.40 ± 4.82
β-Sitosterol-β-o-glucopyranoside	> 50
Hardwickiic acid (3)	21.90 ± 3.50
Bincatriol (4)	35
Crolechinic acid (6)	25
Korberin A (7)	25
Korberin B (8)	25

was greatly reduced (187 μg/ml). The IC_{50} values for various extracts of the sap were similar to that for the dried sap, although the EtOAc extract had a slightly smaller value. Amongst the methanol extracts of the other parts of the plant, the heartwood extract has the smallest IC_{50} value (25 μg/ml). These results clearly show that neither the sap nor the crude extracts are cytotoxic.

Individual compounds from the sap were then examined. Compared to emetine hydrochloride, the control (IC_{50} = 0.2 μg/ml), none of them was highly active. The IC_{50} values for flavanols and proanthocyanidins, the major constituents of the sap, were found to vary considerably, but all of them were greater than 35 μg/ml (Table 1), indicating that they were not cytotoxic. The most active component was 1,3,5-trimethoxybenzene (IC_{50} = 7.13 μg/ml), but the remaining ones obtained from the $CHCl_3$ extract were not cytotoxic, their IC_{50} values being generally greater than 20 μg/ml.

In an earlier study (7), taspine (1) has been shown to be highly cytotoxic to both KB cells and V-79 cells, with IC_{50} values of 0.39 and 0.17 μg/ml, respectively. This compound was derived from the sap of *Croton draconoides* (also known as *C. palanostigma*) of Peru, and was one of the major constituents of the sap (yield 1 % of dried sap). In another study (1), taspine was obtained from the *C. draconoides* sap of Peru in a much greater yield (> 2 %). It then seems likely that the presence of taspine in those saps in such a large quantity may give rise to the anti-tumour activity. However, as observed in our earlier study, the content of taspine varied from one sap to another, and it was present only as a very minor constituent in the *C. lechleri* sap of Ecuador (9). These findings suggest that if the sap of low taspine content has any anti-tumour activity, this may derive from the mechanisms other than inhibiting the growth of the tumour cells, for example, stimulation of the immune system.

Anti-bacterial activity

In this study, the bioautographic TLC assay developed by Hamburger and Cordell (12) was adopted for the evaluation of the anti-bacterial activity. This method proves to be easy to operate, reproducible, and reliable. For each sample, a series of spots with varying concentrations were made on a silica gel TLC plate. The amount of the sample on the spot on which a detectable inhibition to the growth of the bacteria was observed is defined as the minimum inhibition amount (IA_{min}). This value for a variety of samples is listed in Table 2.

The dried sap was active against both bacteria only at higher concentrations (> 10 μg). The MeOH extract showed a similar activity, but the EtOAc and acetone extracts were less active. The $CHCl_3$ extract displayed a considerable inhibition to the growth of *B. subtilis*, although it was less active against *E. coli*.

The polyphenolic compounds derived from the sap were found not to be highly active, and the minimum inhibition amount for them was generally greater than 25 μg. However, several constituents from the $CHCl_3$ extract (9), such as 1,3,5-trimethoxybenzene and 2,4,6-trimethoxyphenol, were found to be extremely active against *B. subtilis*, and their IA_{min} values were 0.0003 μg, 30-fold as potent as penicillin and chloramphenicol. They were also highly inhibitory to *E. coli*. Amongst the six diterpenoids, korberin A (7) and korberin B (8) (both having a lactone functionality) showed a great activity against *B. subtilis* (0.04 and 0.05 μg, respectively). Crolechinic acid (6) was also active against both bacteria. It is

Table 2 Anti-bacterial activity of the sap (minimum inhibition amount).

Sample	*Bacillus subtilis* (μg/spot)	*Escherichia coli* (μg/spot)
Penicillin V	0.008	2.0
Chloramphenicol	0.008	0.008
Sap (freeze dried)	10	10
$CHCl_3$ Extract	0.08	10
EtOAc Extract	50	50
Acetone Extract	30	30
MeOH Extract	10	10
1,3,5-Trimethoxybenzene	0.0003	0.04
2,4,6-Trimethoxyphenol	0.0003	1.0
4-Hydroxyphenethyl alcohol	> 25	> 25
β-Sitosterol	> 25	> 25
Crolechinol (5)	25	5.0
Crolechinic acid (6)	0.2	1.0
Korberin A (7)	0.04	25
Korberin B (8)	0.05	25
(+)-Catechin	25	25
(+)-Gallocatechin	5.0	25
(−)-Epicatechin	25	25
(−)-Epigallocatechin	50	25
Procyanidin B-4	> 25	> 25
Procyanthocyanidin trimer	> 25	> 25
Procyanthocyanidin tetramer	> 25	> 25

of interest to note that the $CHCl_3$ extract and those highly active constituents displayed a high selectivity towards *B. subtilis*.

The presence of these active constituents helps justify the use of the sap as an anti-infectious agent. The major constituents of the sap, proanthocyanidins were slightly active towards both bacteria, but they may contribute to a significant extent to the anti-infectious activity of the sap. Their inhibitory activity towards a wide range of microorganisms is well-known (13).

Effect on proliferation of endothelial cells

The wound-healing process involves several major stages, including coagulation, inflammation, formation of granulation tissue and matrix formation, and remodelling (14). Each of them involves very complex biological processes. During the formation of new tissue, endothelial cells proliferate and form new blood vessels.

In this study, the wound-healing property of the sap was evaluated by examining its effect on the proliferation of endothelial cells. The *in-vitro* assay used measured the incorporation rate of ^3H-thymidine into the DNA of the cells in the presence of the test samples. This rate was used as an indicator of the DNA synthesis and proliferation of the cells, and expressed as a percentage of that measured for the blank control. Therefore a rate of greater than 100% means a stimulation effect, while a rate of less than 100% an inhibitory effect. The results are presented in Table 3.

Table 3 Incorporation rate of ^3H-thymidine into DNA of endothelial cells in the presence of the sap.

Sample	Concentration (μg/ml)	Incorporation rate (% of the blank control)
Dried sap	20	68 ± 12
$CHCl_3$ Extract	20	60 ± 2
EtOAc Extract	20	78 ± 7
Acetone Extract	20	100 ± 4
MeOH Extract	20	88 ± 5
1,3,5-Trimethoxybenzene	0.5	0.1 ± 0.1
4-Hydroxyphenethyl alcohol	10	98 ± 7
β-Sitosterol	10	89 ± 4
Korberin A (7)	10	51 ± 3
(+)-Catechin	10	97 ± 7
(+)-Gallocatechin	5	110 ± 3
(−)-Epicatechin	10	98 ± 1
(−)-Epigallocatechin	5	105 ± 6
Procyanidin B-4	10	108 ± 1
Procyanthocyanidin trimer	20	81 ± 6

The dried sap was found to be very inhibitory to the ^3H-thymidine incorporation (68%). The acetone extract showed no effect, but the other crude extracts were slightly inhibitory, in particular the $CHCl_3$ extract, which displayed a considerable inhibition (60%).

Examination of individual compounds derived from the $CHCl_3$ extract (9) showed that 4-hydroxyphenethyl alcohol had no effect and β-sitosterol was slightly inhibitory; korberin A (7) showed a marked inhibition; and 1,3,5-trimethoxybenzene was extremely inhibitory. Several polyphenolic compounds, such as gallocatechin, epigallocatechin, and procyanidin B-4 slightly stimulated the cell proliferation, whilst the others such as catechin and epicatechin showed little effect. Proanthocyanidin trimer was slightly inhibitory.

These observations show that the Ecuadorian sap investigated in the present study is generally an inhibitory agent to the proliferation of the endothelial cells. In this context, it is of interest to note that although 3',4-*O*-dimethylcedrusin (2) has been reported to be the wound-healing active principle of Peruvian sap, it did not stimulate proliferation of endothelial cells, and instead it did protect the cells against degradation in a starvation medium (6). An *in-vivo* study has shown that the polyphenolic fraction of Peruvian sap is effective in wound healing in rats (16). It was observed that, after such treatment, the tissue contraction on the wound occurred after one day, and the wound site was completely covered with a dark crust. Microscopic evaluation made four weeks later revealed that the new tissue was well formed such that there was no difference between it and the undamaged tissue. But this healing effect was not observed with the rats treated with taspine or 3',4-dimethylcedrusin. This finding confirms the significant role of polyphenolic compounds in the wound healing process.

The distinctive feature of polyphenolic compounds is to bind to a variety of biomacromolecules, such as proteins and enzymes, leading to precipitation (16). It is generally accepted that the anti-microbial and anti-viral activities of polyphenols result from this binding which may inactivate various enzymes involved (17).

In order to explain the wound-healing activity of the sap it is observed that it forms an occlusive film. Within this protective layer the polyphenols prohibit external microbes from attacking the wound. The antibacterial constituents of the sap may make additional contribution as do the anti-inflammatory and the free radical scavenging activity due to proanthocyanidins (18).

The sap used in the present investigation was obtained from Ecuador and it contained only traces of taspine whilst previous investigations of Peruvian sap have reported the taspine content of > 1% (7, 13). In view of the cytotoxic activity of taspine, saps with high taspine content should not be recommended for the treatment of wounds or for internal use. The variations in chemical composition between Dragon's blood of different origins is considerable and for medicinal use quality assurance procedures need to be set up in the countries where these saps are used.

Acknowledgements

The authors wish to thank the Körber Foundation of Germany for financial support as part of 1989 Award for Achievements in European Science. We are grateful to Professors M. H. Zenk (Universität München) and Y. Y. Gleba (Academy of Science, Kiev) for providing the plant materials and to Marion Dawson for technical assistance with the antibacterial testing.

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Efficacy of Crofelemer (SP-303) in Patients with Diarrhea

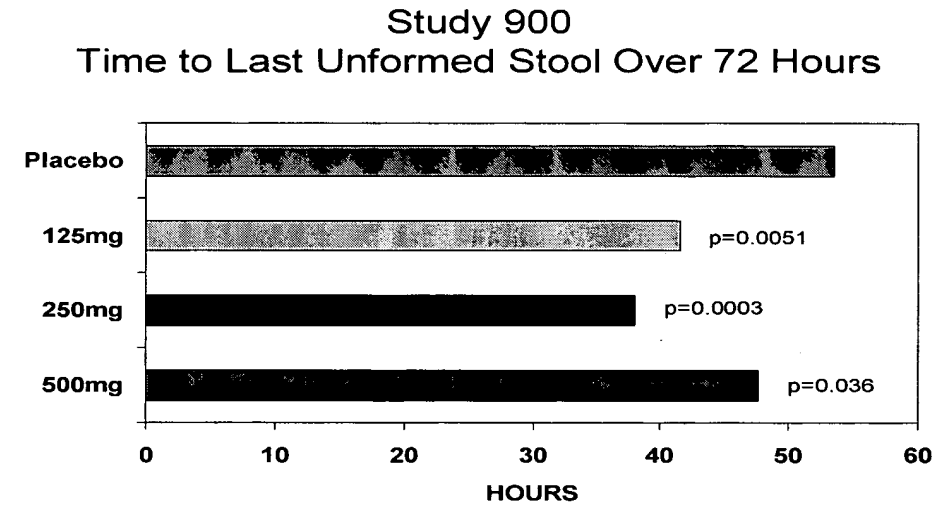
Based on its mechanism of action, i.e., blocking chloride ion secretion through the cystic fibrosis transmembrane conductance regulator (CFTR), crofelemer (previously known as SP-303) was investigated as an agent for the treatment of secretory diarrhea. Crofelemer was evaluated in patients with Traveler's diarrhea (most linked to enteric infections with enterotoxigenic *E. coli* [ETEC]) and HIV-associated diarrhea, which is a more diverse group of patients.

The efficacy data obtained in patients with HIV-associated diarrhea was sufficient to obtain Fast-Track status from the FDA in 1998 and to have that status reaffirmed in a meeting with the FDA in April 2004.

Traveler's diarrhea

A total of 184 patients with traveler's diarrhea or non-specific diarrhea were enrolled in a Phase 2, double-blind, placebo-controlled dose-ranging trial in which crofelemer was given at a dose of 125 mg, 250 mg, or 500 mg qid for 2 days; crofelemer was administered as enteric-coated tablets in capsules. Compared to placebo, all three doses of crofelemer produced a statistically significant beneficial effect, including earlier recovery time (Figure 1), increased number of patients experiencing recovery by 24 hours of treatment (Figure 2), reduction in overall treatment failure rate (Figure 3), and improved symptoms, including abdominal pain and urgency. The results from this study clearly demonstrate the activity of SP-303 and support its use in secretory diarrhea.

Figure 1 Time to last unformed stool over 72 hours in phase 2 Traveler's diarrhea study 900



Mean values

Figure 2 **Partial of complete improvement at 24 hours in phase 2 Traveler's diarrhea study 900**

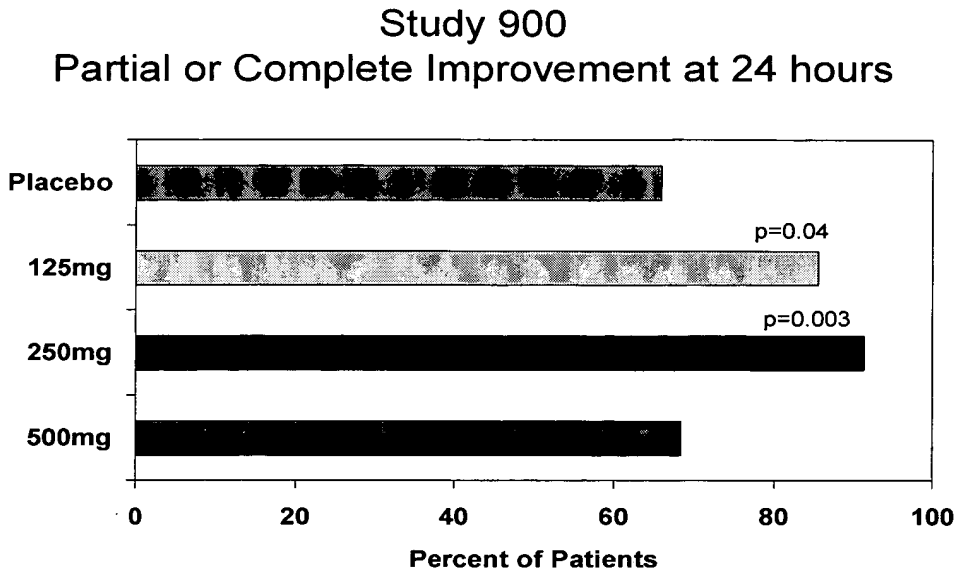
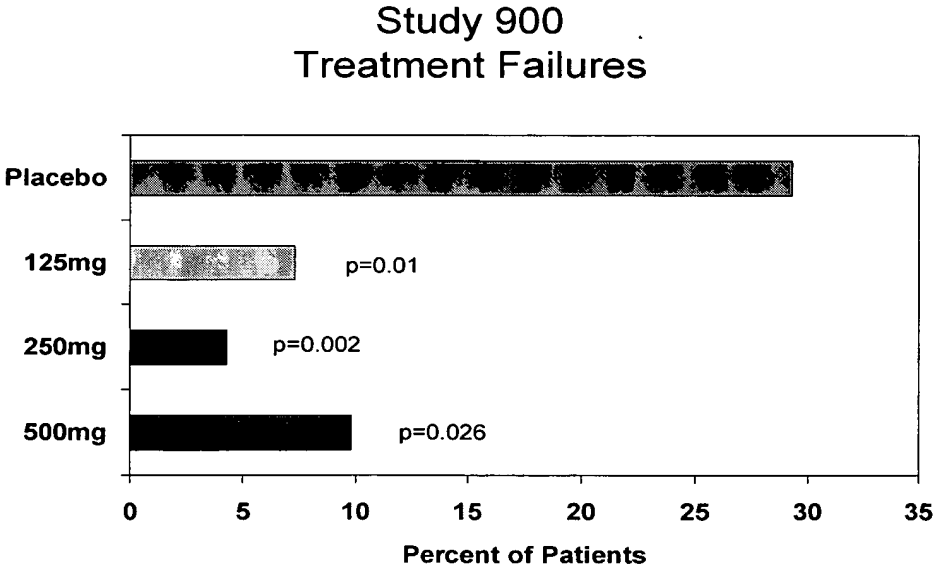


Figure 3 **Treatment failures in phase 2 Traveler's diarrhea study 900**

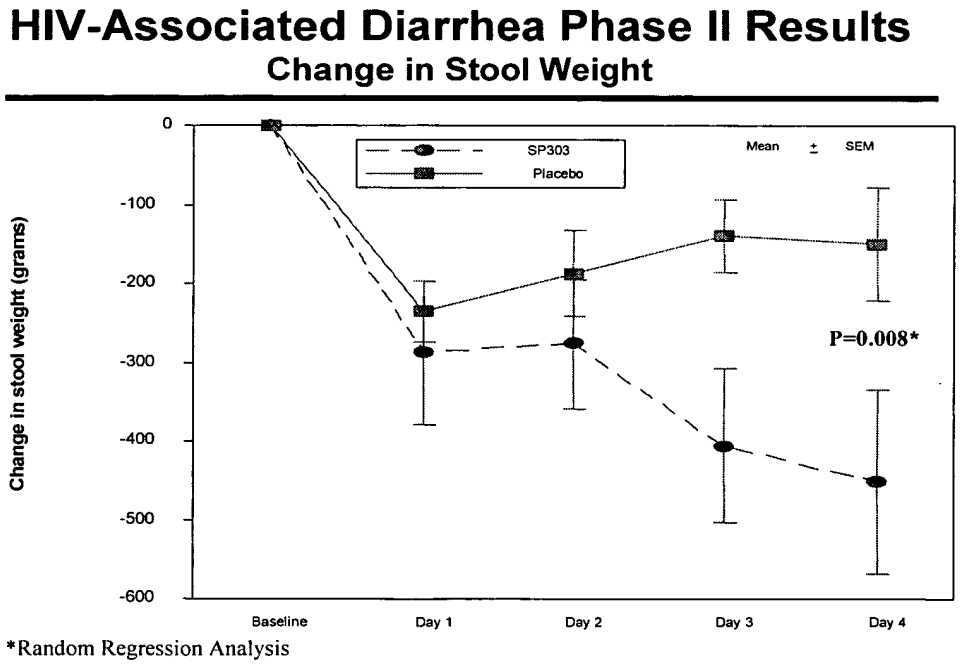


HIV-associated diarrhea

Two studies were conducted with crofelemer in patients with chronic diarrhea associated with HIV/AIDS. In both trials, crofelemer produced clinically relevant decreases in stool weight and decreased stool frequency; significant improvements in gastrointestinal symptoms, particularly urgency, were also associated with crofelemer therapy. Significant reductions in stool chloride concentrations were noted in patients receiving crofelemer, confirming its preclinical mechanism of action.

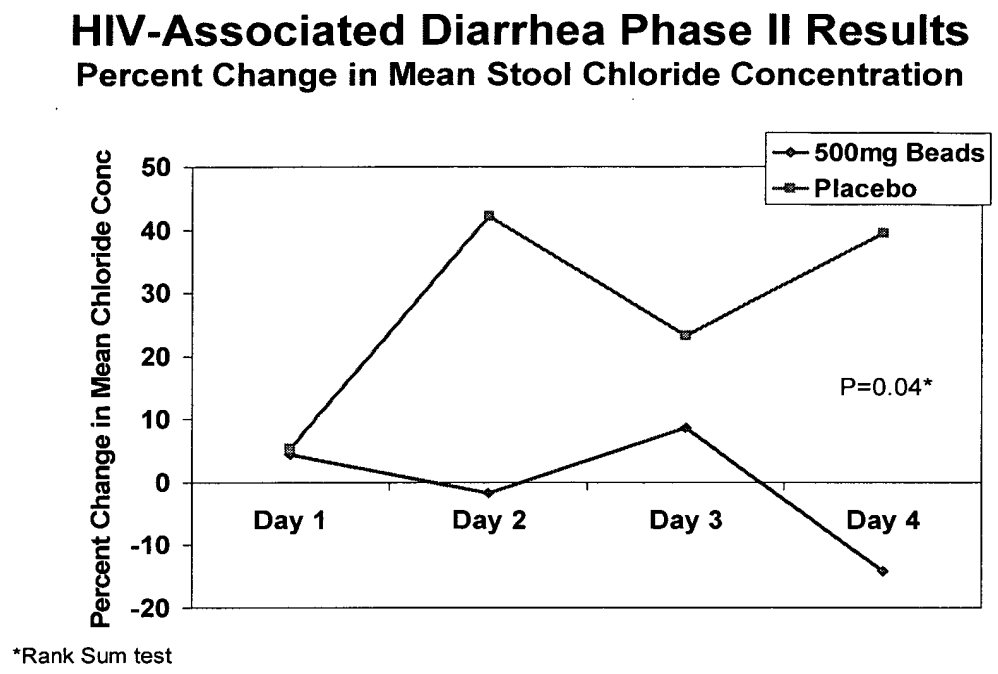
Croflemer was previously evaluated in a phase 2 study in patients with HIV-associated diarrhea entitled "A Double Blind, Randomized, Placebo Controlled, Phase II Study to Assess the Safety and Efficacy of Orally Administered SP-303 for the Symptomatic Treatment of Diarrhea in Patients with AIDS." An enteric coated bead formulation of crofelemer was given at doses of 500 mg qid for 4 days in a hospital inpatient setting. Crofelemer produced a substantial reduction in daily stool weight and frequency by Day 4 (Figure 4). A random regression analysis of slopes revealed statistically significant differences in both stool weight (p=0.008) and in stool frequency (p=0.04) compared to placebo. Significant reductions in stool chloride concentrations compared to placebo were noted in patients receiving crofelemer (Figure 5), confirming its preclinical mechanism of action.

Figure 4 **Change in stool weight in study 37,554-209 (SP-303 500 mg beads versus placebo)**



*Random Regression Analysis

Figure 5 **Percent change in mean stool chloride concentration in study 37,554-209 (SP-303 500 mg beads versus placebo)**



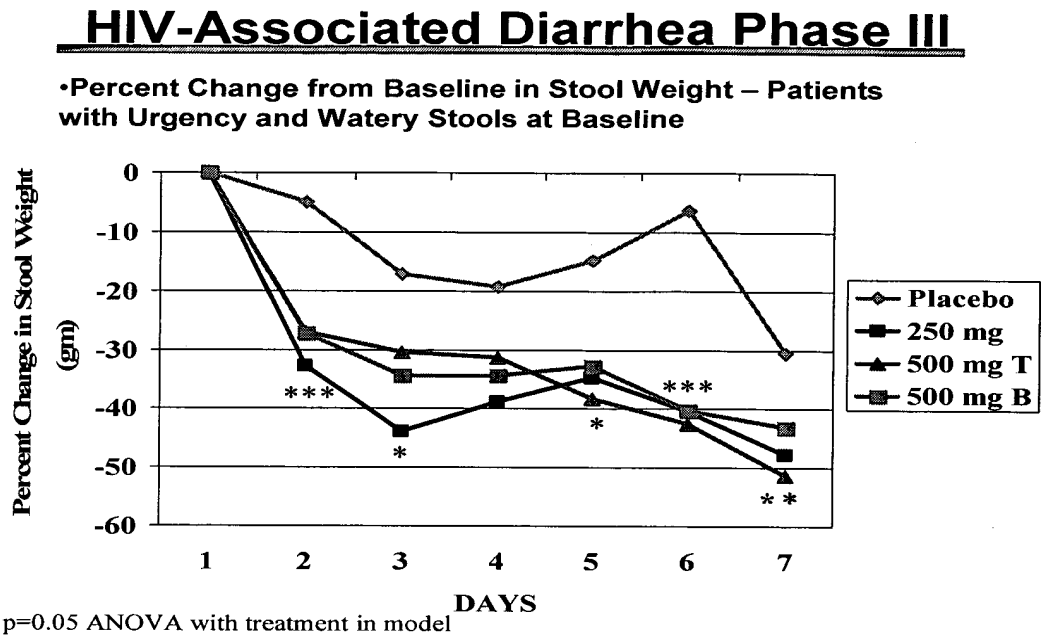
*Rank Sum test

The significant efficacy observed in this phase 2 trial led to a phase 3 study, "A Double Blind, Randomized, Placebo Controlled, Phase III Study to Assess the Safety and Efficacy of Orally Administered SP-303 for the Symptomatic Treatment of Diarrhea in Patients with AIDS." In this study, 400 patients with chronic HIV-associated diarrhea were treated with crofelemer or placebo. Crofelemer was given at doses of 250 mg and 500 mg enteric-coated tablets or 500 mg enteric-coated beads in capsules qid for 6 days in an inpatient setting; patients who responded to treatment were continued in a four-week blinded out-patient phase. In a random regression analysis, the difference between the 500mg tablet group and placebo was significant ($p = 0.03$). Analysis of patients who presented with several watery stools at baseline or who presented with at least one watery stool and an important gastrointestinal symptom demonstrated statistically significant efficacy for crofelemer treatment.

Statistically significant efficacy of crofelemer is also observed in a subset of patients in the ITT population with at least one watery stool and some associated urgency at baseline defined as the "significant baseline diarrhea subset." Urgency was prospectively selected as an important marker of truly symptomatic patients, and was one of the most frequently reported gastrointestinal symptoms in the study. In the patients with watery stools and urgency at baseline, significant reductions in percent change in total stool weight (Figure 6) and abnormal stool weight were observed. In both of these analyses, significant differences between active and placebo arms were observed starting at Day 2. Significant reductions in the frequency of total stools and abnormal stools were also observed in the significant baseline diarrhea subset. The number of patients who continued to experience watery stools on Day 7 was also significantly lower for the 500mg dose arms compared

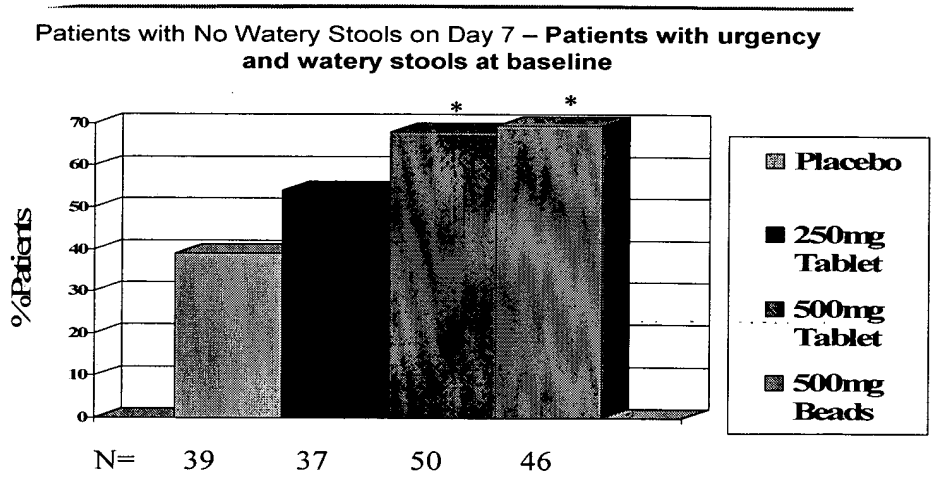
to placebo (Figure 7); confirming that both formulations, which protect crofelemer from the stomach environment, produced equivalent efficacy.

Figure 6 **Percent change in stool weight in significant baseline diarrhea subset in study 37,554-210**



* p=0.05 ANOVA with treatment in model

Figure 7 **Patients with <u>no</u> watery stools on Day 7 in significant baseline diarrhea subset in study 37,554-210**



* $p \leq 0.02$; Shaman ITT population